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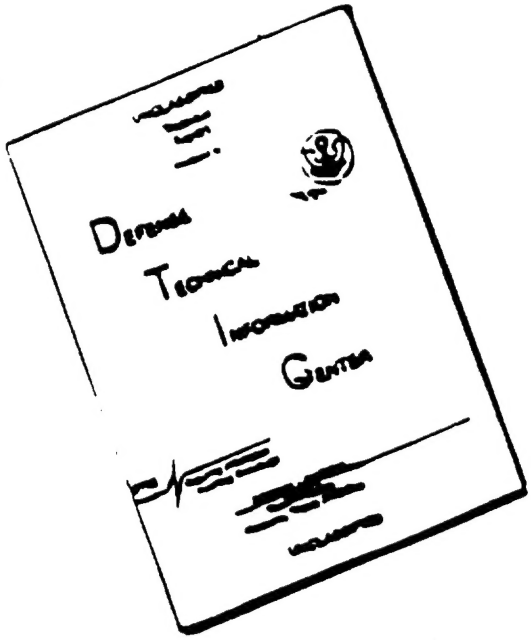

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In order to continue to determine the degree to which the accumulation of mutations in breast cancer cells is due to a change in the fidelity of the cellular DNA replication machinery, we have continued and expanded our experiments utilizing the multiprotein DNA synthesome isolated from breast cancer cells and normal breast cells. The isolated DNA synthesome is fully competent to replicate template DNA *in vitro* and has been demonstrated by our lab to accurately depict the DNA synthetic process as it occurs *in vivo*. We have used the DNA synthesome from two breast cancer cell lines and two non-malignant breast cell lines to replicate a target gene in an *in vitro* DNA replication assay. By utilizing the target gene in a bacterial mutant selection assay we have determined the frequency with which mutational sequence errors occur as a result of the *in vitro* DNA replication mediated by the breast cancer cell DNA synthesome and the non-malignant breast cell synthesome. Our results suggest that the fidelity of DNA replication mediated by the breast cancer cell synthesome is lower than the fidelity of the non-malignant breast cell synthesome. To date, we have also identified nucleotide insertions, deletions, misincorporations, and transversions in the target gene replicated by the DNA synthesome of a malignant breast cell line. Additionally, we have also initiated experiments designed to elucidate the contribution of DNA nucleotide excision repair to the overall DNA fidelity of these breast cell lines.

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## Introduction

To date it has been nearly impossible to distinguish whether the cellular transformation process gives rise to conditions that cause cancer cells to accumulate mutations (2) , or whether an increase in the rate of mutation within cells gives rise to the transformation process that culminates in the formation of cancer cells (3,4). Currently, a strong case can be made relating the number of unrepaired mutations in the cell with the development of a cancerous phenotype (5-7). The mutations noted to correlate with the expression of a cancerous phenotype could result from an increase in errors made during both DNA replication and DNA repair (8-17). In order to begin to assess the degree to which errors created during DNA replication contribute to the overall mutation frequency observed in cancer cells, I proposed to compare the fidelity of the DNA replication process in malignant breast cells and normal breast cells. Several studies have reported that the activity of DNA β polymerase, an enzyme implicated in gap filling DNA synthesis during DNA repair (18,19) is decreased in cancer cells. These and other investigations also indicate that cancer cells generally have a higher error rate during the repair of gapped DNA and report that at least some of the common mutations include frame shifts and deletions of DNA sequence (17-22). However, most of these investigations were performed using either crude cell extracts or purified enzymes. *In vitro* assays using crude cell extracts contain nucleases and proteases, which may alter the integrity of the replication or repair enzymes in the extract or the DNA templates used in these assays. These factors may subsequently affect our interpretation of the data obtained using crude cell extracts. Those studies that use purified enzymes do not take into account that DNA repair in intact cells generally occurs in a highly controlled environment (13), with both the DNA and key enzymes organized into higher-order structures. These assays also do not adequately consider the potential contributions of accessory factors present in the intact cell that may enhance the fidelity of the DNA repair process. The observations reported by Kunkel's group (18,19) reinforce the idea that the maintenance of high fidelity DNA synthesis and repair requires at least some of the proteins used during DNA replication. Assays that ignore the possible involvement of the DNA replication proteins in the repair process are not capable of presenting an accurate picture of intact cell DNA repair, and also ignore the possible role played by the fidelity with which DNA replication is initially carried out and the overall contribution of the fidelity of DNA replication to the development of a "mutator" phenotype.

In order to better understand the extent to which the intact DNA replication machinery contributes to the overall mutation frequencies observed in normal and malignant breast cells, I have designed experiments to examine the degree of fidelity exhibited during the DNA replication process in both normal and cancerous breast cells. To accomplish this goal I have isolated a multiprotein DNA replication complex (which we have designated the DNA synthesome) from both normal breast tissue cells and malignant breast cancer cells and have begun to determine the ability of the DNA synthesome from both cell types to faithfully copy a target gene used in our *in vitro*

replication assay system. We have previously shown that the DNA synthesome isolated from mammalian cells is fully competent to carry out large T-antigen-dependent DNA synthesis *in vitro* (23-25). The DNA synthesome has been purified to about 30-40 polypeptides and is fully competent to replicate DNA bidirectionally from a defined origin of DNA replication, producing semi-conservatively replicated DNA. The rate of DNA replication and the products of the *in vitro* reaction suggest that the DNA synthesomefaithfully mimics the replication process carried out in intact cells. Using this multiprotein DNA replication complex we have initiated experiments intend to determine whether cancer cells exhibit a higher mutation frequency due to a defect in the fidelity of the DNA synthetic process. Our data suggest that there is a higher frequency of mutation in our assay when the target DNA sequence is replicated by the DNA synthesome from the breast cancer cells than when it is replicated by the DNA synthesome from normal breast cells. Our most recent data suggest that the types of mutations created by malignant breast DNA synthesome-mediated replication may include GT mismatches and nucleotide insertions (G).

Although we have observed decreased DNA replication fidelity of the human breast cancer cell lines in our in vitro system, it is important to also consider the potential contribution of the extensive DNA repair activities present in the intact cell. In order to begin to account for the contribution of the DNA repair systems to the overall picture of DNA replication fidelity in our breast cancer cell lines, we have utilized a nucleotide excision repair assay. To date we have measured the relative ability of the MCF 7 and MCF 10A cell lines to repair and restore the expression of a UV-damaged template containing the gene for chloramphenicol acetyl transferase (CAT).

**Materials and Methods**

a. The pBK-CMV vector from Stratagene (see figure 3 in appendix) contains the full 298 bp SV40 origin of DNA replication, including both large T-antigen binding sites I and II. It also contains the eucaryotic promoter for the cytomegalovirus (CMV), the procaryotic RNA start sequence (at position 1221), the *lac*P gene (at position 1300-1220), which codes for the *lacZ* gene promoter, the α-*lacZ* gene (at position 1183-810), the start site for the β-gal gene (ATG) (at position 1183), and the stop site for the gene (TAA) (at position 799).

b. The XL1- Blue MRF' strain of E. coli: This strain was purchased from Stratagene as the optimal strain of E. coli for the growth and expression of the pBK-CMV vector. Electrocompetent XL1- Blue MRF' cells are purchased and transfected by electroporation (31,32).

c. Expression of the non-mutated β-galactosidase gene in the transformed E.coli growing in the presence of both the chromogenic substrate of the β-galactosidase gene product, 5-bromo-4-chloro-3-indolyl β-D galactoside (X-gal), and the inducer for the β-galactosidase gene, isopropylthio-β-D galactosidase (IPTG), produces dark blue colonies. Errors in the sequence encoding the β-galactosidase gene result in white colonies. Intermediate phenotypes (light blue) may result from less severe mutations of the gene encoding β-galactosidase.

**Cell culture.** The malignant breast cancer cell line, Hs578T (Homo sapiens No. 578, tumor cells), is an aneuploid, mammary epithelial cell line derived from a mammary tumor that does not express the estrogen receptor protein (26). The genetically matched non-malignant breast cell line Hs578Bst (Homo sapiens No. 578, breast cells) is diploid and is, most likely, of myoepithelial origin (26,34). It is derived from breast tissue found peripheral to the Hs578T tumor. The second human breast cancer cell line, MCF7, is derived from a human breast adenocarcinoma. The cells display charcteristics of differentiated mammary epithelium including the ability to process estradiol via cytoplasmic estrogen receptors. The MCF10A cell line (non-malignant) is derived from a mammary gland of a patient with fibrocystic disease. The cells express characteristics of luminal ductal cells. All cell lines were purchased from the ATCC.

The Hs578T cells are grown in suspension in Dulbecco's modified Eagle's medium supplemented with 4.5 g/L glucose, 10 units /ml bovine insulin, and 10% fetal bovine serum (FBS). The Hs578Bst cells are grown in monolayer culture with modified Dulbecco's medium, 30 ng/ml epidermal growth factor (EGF), and 10% FBS. The MCF 7 cells are propagated in Eagle's MEM with non-essential amino acids, 1 mM sodium pyruvate, 20μg bovine insulin/ ml, 90%, fetal bovine serum, 10%. The MCF10A cells are grown in monolayer culture with modified Dulbecco's medium and Ham's F12 medium with 20ng/ml epidermal growth factor (EGF), 100ng/ml cholera toxin, 0.01mg/ml insulin, and 500ng/ml hydrocortisone, 95%, horse serum, 5%.

**Fractionation scheme for the Isolation of Breast Cell Multiprotein DNA Replication Complex (the DNA synthesome).** Hs578T or MCF7 breast cancer cells (26) and Hs578Bst or MCF10A non-malignant breast cells (26) are collected by low-speed centrifugation, and the cell pellet resuspended in PBS and frozen at -80°C using the procedure previously described by our laboratory (28,35,36). The human breast cell DNA synthesome are isolated using the procedure described by Malkas et al. (see figure 1 in appendix) (35). Briefly, frozen Hs578T or MCF7 breast cancer cells (26) and Hs578Bst or MCF10A non-malignant breast cell (26) pellets are thawed and resuspended in buffer, homogenized, and then centrifuged at 2,500 rpm (1,740 x g ) for 10 minutes in order to separate the crude nuclear (P-1) and cytosolic fractions (S-1). Mitochondria (P-2) are pelleted from the S-1 fraction by centrifugation at 12,500 rpm (18,000 x g) for 15

minutes. The resultant supernatant (designated the S-2) fraction is then subjected to ultracentrifugation at 100,000 x g for 1 hour to remove microsomes (P-3), and the supernatant are designated the S-3 fraction. The crude nuclear pellet (P-1) are resuspended in buffer and gently rocked for 2 hours. After a 10-minute centrifugation at 15,000 x g the supernatant (designated NE), containing soluble protein extracted from the nuclei, is collected, combined with the S-3 fraction and made 2M in KCl and 5% in polyethylene glycol (PEG 6000). The mixture is stirred gently for one hour at 4°C and pelleted by centrifugation for 15 minutes at 16,000 rpm (30,900 x g). The resultant supernatant (PEG NE/S-3) is collected and layered onto a 2M sucrose cushion and subjected to centrifugation at 40,000 rpm (100,000 x g) for 16-18 hours at 4°C. The material above the sucrose cushion (top 80% of the tube) is collected and designated the S-4 fraction. The material collected at the sucrose interface is designated the P-4 fraction.

The P-4 fraction is then applied to a Q-Sepharose column (Pharmacia) (25 mg protein/1 ml of matrix) which is pre-equilibrated with loading buffer containing 50 mM Tris-HCl, pH 7.5 / 1 mM DTT / 1 mM EDTA / 10% glycerol / 50 mM KCl. Unbound protein is washed from the matrix with 8 volumes of column-loading buffer. The matrix-bound protein is eluted by an increasing KCl gradient (50 mM - 1 M) in 50 mM Tris-HCl, pH 7.5 / 1 mM DTT / 1 mM EDTA / 10% glycerol. The column fractions will then be assayed for their ability to support *in vitro* SV40 DNA replication. The column fractions able to support *in vitro* SV40 DNA replication are pooled and layered onto an 11-ml 10-30% sucrose gradient containing 50 mM Tris-HCl, pH 7.5 / 1 mM DTT / 1 mM EDTA / 0.5 M KCl. The tubes containing the sucrose gradients are centrifuged at 100,000 x g for 16 hours, and the sucrose gradient fraction containing the replication-competent DNA synthesome is aliquoted and stored at -80°C.

***In Vitro* DNA Replication Assay.** The reaction mixtures (50 µl) contain 30 mM HEPES (pH 7.8), 7 mM $MgCl_2$, 0.5 mM DTT, 50 µM dATP, 100 µM each of dGTP, dCTP, dTTP, 5 µCi [$\alpha$-$^{32}$P]dCTP (4,000 cpm/pmol), 200 mM each of CTP, UTP, GTP, 4 mM ATP, 40 mM phosphocreatine, 100 µg of creatine phosphokinase, 15 mM sodium phosphate (pH 7.5), 30 ng of the plasmid pBK-CMV, and approximately 1.0 µg of large T-antigen (3,23). The reaction mixture is incubated at 37°C for 3 hours. After 3 hours, 10 µl of the reaction mixture is pipetted onto Whatman DE81 filters and allowed to air-dry. The filters are then washed 1 x (5 min.) with 0.1 M NaPPi buffer (pH 7.0) , and 3 x (5 min.) with 0.3 M ammonium formate (pH 7.4), air-dried, and counted in a liquid scintillation counter.

**Precipitation of the Replicated DNA.** The DNA plasmids in the remaining 40 µl from each *in vitro* DNA replication reaction are extracted with phenol/chloroform /isoamyl (2 x 260 µl), followed by extraction with chloroform/isoamyl (25: 1) (2 x 160 µl). They are then precipitated with 0. 2M ammonium acetate and 100% isopropanol. After centrifugation at 12,000 rpm (14,956 xg), and three washes of 70%

ethanol, the resulting pellets are dried and then each resuspended in 260 µl TE buffer (10 mM Tris-HCl, pH 8.0 / 1 mM $Na_4$ EDTA), and subjected to Dpn I digestion.

**Analysis by Gel Electrophoresis of Replicated DNA Products.** Approximately 2-3 µl of the Dpn I-treated newly replicated DNA from each reaction mixture are mixed with 5 µl of a sodium-dodecylsulfate (SDS)-containing dye mix (20 mM Tris-HCl, pH 8.0 / 5 mM EDTA / 5% SDS / 0.5% bromophenol blue / 25% glycerol). The pBK-CMV DNA are size fractionated by electrophoresis through a 0.8% agarose gel matrix at 40 V for 16 hours. The agarose gel matrix are prepared using Tris/glacial acetic acid/EDTA (TAE) buffer and 0.5 mg/ml ethidium bromide. The DNA products are visualized using autoradiography.

**Forward Mutagenesis Assay: Transfection and Plating.** (see figure 5 in appendix) The *in vitro* replicated pBK-CMV DNA from the Dpn I digestion will be used in the transfection of the E. coli host. Electrocompetent bacteria (E. coli strain XL1- Blue MRF') are mixed with approximately 300 pg of pBK-CMV DNA isolated from the *in vitro* DNA replication reaction, diluted to 40 µl total volume with 10% glycerol in LB media, incubated for 10 min. on ice, and electroporated (1.4 kV, 25 µF, 200 ohms). Immediately following the electroporation, 960 ml of chilled sterile SOC buffer (20mM glucose in LB media) are added to the cuvette. The electroporated mixture is then incubated at 37°C for 1 hour at 250 rpm. An amount of the culture sufficient to yield 100-600 bacterial colonies per plate are plated onto 20 ml LB agar containing 0.5 mg/ml kanamycin, 25 mg/ml IPTG and 25 mg/ml X-gal . These plating conditions give intense blue color for the wild-type plasmid which facilitates the visualization of mutant phenotypes. The mutant colonies range from white to intermediate (relatively blue) phenotypes.

**Scoring of Mutants.** The inactivation of the α-complementation gene (the product of which is the catalytic subunit of β-galactosidase) due to a mutation in the *lac* Zα gene in pBK-CMV will give a variety of mutant phenotypes, due to the lack of a fully functional β-galactosidase gene product. These mutant phenotypes are scored after approximately 12-15 hours of incubation at 37°C. In order to reproducibly and precisely score the variable color intensity mutant phenotypes, a scale of blue color intensities has been established (1). Using the plating conditions described above, the wild-type pBK-CMV DNA generates a dark blue phenotype, which on a scale of 0-4, are assigned a value of 4. The variable mutant phenotypes can be distinguished as 0+ (white/colorless), 1+ (faint blue), 2+ (medium blue), or 3+ (almost wild type). To eliminate false positives we count only the pure white (0+ phenotype) colonies in our calculation of the percent mutant colonies per plate.

Since the background mutation rate for a *lacZα* forward mutation assay employing M13 phage is typically about 2-5 x $10^{-4}$, we have chosen a phagemid DNA, pBK-CMV,

for which no detectable mutation rate has been demonstrated (Weeks and Hickey, unpublished data).

**Determination of the nucleotide sequences of the DNA replicated *in vitro*.** Double-stranded replicated DNA from the bacterial mutants selected as just described are isolated using the Bio 101 Plasmid Isolation Kit (each scored mutant phenotype separately) and sequenced by the dideoxynucleotide chain termination method as described by Sanger (29). Two individual oligonucleotides will prime the reactions for sequence analysis (see figure 9 in the appendix). Sequencing reactions using these two primers will span a region of 373 bp.

**DNA nucleotide excision repair assay.** The protocol of Dr. Lawrence Grossman (37) was followed. Briefly, the breast cells were seeded at 2.0 x $10^6$ cells in 12 x 75 mm dishes. Deae/dextran transfection was used to introduce 5 µl of 50 µg/ml of UV damaged (350 or 700 J/$m^2$) or undamaged (control) template plasmid DNA(pCMV*cat*) into the cells. The cells were allowed to grow and repair the DNA over a 40 hr. period. After the incubation, a cell free extract was prepared from the cells. The presence of the enzyme chloramphenicol acetyl transferase (CAT), expressed from a correctly repaired pCMV*cat* plasmid, will catalyze the reaction of chloramphenicol and $^3$H-acetyl coenzyme A to yield acetylchloramphenicol-$^3$H. After extraction of the reaction with ethyl acetate, the acetylchloramphenicol-$^3$H is counted via liquid scintillation. The level of DNA repair is expressed as % CAT activity remaining after the plasmid is presented with a defined dose of UV (J/$m^2$) and allowed to incubate inside the transfected cell. All results are compared to the results of the positive control (normal peripheral blood lymphocytes) and the negative control (nucleotide excision repair deficient XPC cell line). Each condition was performed in triplicate.

## Results

### I. Results of Task I : Preparation of the replication template DNA, months 1-12.

Since submitting the original proposal, in which we describe using the M13mp2 bacteriophage as the DNA template for the replication assay, we have discovered that M13mp2 bacteriophage has an intrinsic mutation rate of approximately 2-5 x $10^{-4}$. In order to minimize the background mutation frequency rate we will use the pBK-CMV plasmid (from Stratagene), for which the detectable mutation rate has been observed to be less than 1 x $10^{10}$ colonies (Weeks and Hickey, unpublished data). As shown in figure 3, the pBK-CMV contains the SV40 origin of replication, including large T-antigen binding sites I and II, and the kanamycin resistance gene. We have successfully grown this plasmid in XL1-Blue MRF' E.coli, a strain selected for its ability to support optimal

growth and expression of this plasmid (also from Stratagene), and isolated and purified the supercoiled form I plasmid DNA for use in the DNA replication assay.

## II. Results of Task 2: Purification of the Multiprotein Replication Complex ( DNA synthesome ) , Months 1-12.

Our laboratory has isolated a multiprotein DNA replication complex (DNA synthesome) from human cervical carcinoma cells (HeLa) (35,36), from mouse mammary cells (FM3A) (28) and most recently, from MDA MB 468 human breast cancer cells (36), from the genetically matched human breast cell lines, Hs578Bst (non-malignant) and Hs578T (cancerous), and from the human breast epithelial cell lines MCF7 (malignant) and MCF10A (non-malignant) (Weeks et al., unpublished data). The complex is isolated using a series of steps that includes ultracentrifugation, polyethylene glycol precipitation, and ion-exchange chromatography, as shown in the schematic figure 1. The sedimentation coefficient of the multiprotein complex from the MDA MB 468 breast cancer cells is approximately 18S as measured by sucrose gradient density analysis (36). The sedimentation coefficients of the Hs578Bst (non-malignant), Hs578T (cancerous), MCF10A (non-malignant), and MCF7 (malignant) DNA synthesomes are currently under analysis in our laboratory. The integrity of the multiprotein complex is maintained after treatment with DNase, RNase, 2M KCl, NP40/butanol, and Triton X-100, and after chromatography on DE52-cellulose and Q-Sepharose, suggesting that the association of proteins with one another is independent of nonspecific interaction with other cellular macromolecular components (28).

Most importantly, we have demonstrated that the DNA synthesomes from the MDAMB 468 (36), and from the Hs578BSt, Hs578T, MCF10A, and MCF7 (Weeks et al., unpublished data), are fully competent to replicate DNA *in vitro* in a variation of the assay described by Li and Kelly (1984) (30). The demonstrated replication ability of the isolated multiprotein form of DNA polymerase suggests that all of the cellular activities required for large T-antigen-dependent *in vitro* papovavirus (i.e., SV40 and polyoma virus) DNA synthesis are present within the isolated DNA replication apparatus. Our lab has previously found that the mammalian DNA synthesome includes DNA polymerase $\alpha$, DNA primase, DNA polymerase $\delta$, proliferating cell nuclear antigen (PCNA), RP-A (a.k.a. RF-A, and HSSB), topoisomerases I and II, and RF-C or Activator 1 (A-1) protein complex (28,35,36). The presence of these enzymes in the DNA synthesome has been verified by both Western blotting and, when possible, enzyme activity assays (e.g., RP-A, RF-C, PCNA do not have intrinsic enzymatic activity). The most current model of the DNA synthesome is shown in figure 2 in the appendix.

**III. Results of Task 3: Isolation and analysis of the DNA synthesome -mediated DNA replication products, months 1-12.**

DNA replication products have been isolated and purified from Hs578Bst and Hs578T, MCF10A, and MCF7 DNA synthesome -mediated in vitro replication assays. The purified DNA product has been subjected to Dpn I digestion and separated on a 1% neutral agarose gel. The products (visualized by autoradiography) demonstrate that the replication reactions mediated by all four cell type DNA synthesomes are capable of producing a full length DNA replication product. The level of DNA replication in each reaction has also been examined by measuring the incorporation of [$^{32}$P]- dCTP into the newly replicated DNA collected on DE81 filters and counted by liquid scintillation counting. Interestingly, the net nucleotide incorporation after a 3 hour incubation period is approximately 1.5 times higher in the replication reactions mediated by the cancerous Hs578T and MCF7 DNA synthesomes than those mediated by the non-malignant Hs578BsT and MCF10A DNA synthesomes.

**IV. Results of Task 4: Transfection and expression of the replicated DNA product in E. coli, months 12-36.**

The XLl-Blue MRF' strain of E.coli was successfully transfected with each of the following : the wild type pBK-CMV, the fully replicated/Dpn I digested pBK-CMV, and an equimolar concentration of pUC19 DNA (negative control) as described in the methods. The entire electroporation mixture containing the transfected E.coli and SOC medium was plated (100 ul per plate) onto LB agar plates containing optimal concentrations of kanamycin, X-gal, and IPTG. The transfected E.coli containing the wild type pBK-CMV expressed a dark blue phenotype (100%). The E.coli transfected with the negative control DNA (pUC19) consistently created mutant (white) colonies (100%). The E.coli transfected with pBK-CMV replicated by the DNA synthesome from the Hs578T (cancerous) cell line demonstrated a mutant (white) phenotype with a frequency of 1.04 % (x = 10.41/1000 colonies). Similarly, E.coli transfected with DNA produced in replication reactions mediated by the MCF7 DNA synthesome resulted in a 1.7% frequency of mutant colonies. Mutant colonies created by the non-malignant Hs578Bst and the MCF10A DNA synthesome-replicated DNA were only found with frequencies of 0.18% (x = 1.84/1000 )(Hs578Bst) and 0.46% (MCF10A) (see figures 6 and 7).

To date, three separate experiments have been completed, under our empirically determined optimal conditions, all of which suggest that the frequency of replication errors in the maligant Hs578T and the MCF7 DNA synthesome -mediated replication reaction appear to be approximately 5.8 times (Hs578T) and 8.9 times higher (MCF7) that of the plasmid replicated by the DNA synthesome from the non-malignant Hs578Bst and MCF10A cells (see figure 8 in the appendix).

Mutant colonies were collected and grown separately in LB broth containing tetracyclin and kanamycin over night. The clonal populations of mutant E.coli were then collected by centrifugation, resuspended in fresh LB broth, made 10% in glycerol, and frozen at -80° C for later extraction and nucleotide sequencing of the mutant plasmid.

## V. Results of Task V: DNA sequencing and analysis of the mutant DNA replication products, Months 12-48.

I have built an extensive library of clonal mutants isolated from the blue/white mutant selection assay. In order to determine whether the mutations observed in the bacterial colonies isolated from the mutant selection assay occur randomly or are located to specific segments of the plasmid DNA template we have begun to sequence β-galactosidase gene (Lac Z) extracted from clonal mutant (white) and normal (blue) colonies. To this date, we have been able to successfully sequence two mutant and one normal plasmid extracted from bacteria transfected with the pBK-CMV replicated by the breast cancer DNA synthesome (MCF7). To sequence the β-galactosidase gene (α lac Z) we use the Sanger dieoxynucleotide sequencing method and flanking primers (27 bp each) shown in figure 9 of the appendix. A scanned image of mutant and normal sequences from the MCF7 DNA synthesome-mediated fidelity assay are shown in figure 10. The types of mutations detected by our forward mutagenesis assay include insertions deletions and G-T and C-T mispairs (figure 11).

## VI. Results of additional experiments: The excision nucleotide DNA repair of the malignant versus the non-malignant human breast cell lines.

As shown in figure 12 of the appendix, the excision nucleotide DNA repair of the MCF 7 cell line was found to be enhanced 25 fold over both the MCF 10A cell line and the "normal" control (peripheral blood lymphocytes- PBL). The negative control is a Xeroderma Pigmentosa cell line (XPC), which is defective in nucleotide excision DNA repair. These results suggest that the excision repair activity of this breast cancer cell line is much greater than normal.

## Discussion

Our original proposal described experiments in which an M13 vector containing the SV40 viral origin of DNA replication and the β–galactosidase gene were covalently linked. This M13 vector was used in an *in vitro* DNA replication assay in which DNA

synthesis was mediated by a multiprotein DNA replication complex (the DNA synthesome) isolated from both malignant and non-malignant human breast cells. Our goals were to determine whether the DNA synthetic machinery (the DNA synthesome) of breast cancer cells was more error-prone than the DNA synthetic machinery of normal breast cells, and whether our results supported the hypothesis that the higher incidence of mutations observed in breast cancer cells, was due to a reduction in the fidelity of the breast cancer cell DNA synthesome relative to the fidelity of the normal breast cell DNA synthesome .

In setting up the mutation selection assay, we discovered that M13 had an inherent mutation frequency of 2-5 x $10^{-4}$. We believed that this high spontaneous rate of mutation, when the M13 was simply transfected into bacterial cells, would potentially mask the true mutation rate arising from errors created by the breast cancer cell DNA synthesome. If this were correct it would make it impossible to accurately assess whether the breast cancer cell DNA synthesome was error-prone. To overcome this potential difficulty we developed a strategy to covalently link the SV40 viral origin of replication and the β-galactosidase gene into a regular plasmid. During our initial planning of the details to construct this plasmid vector, we discovered that Stratagene had already constructed such a vector (pBK-CMV), and that it could be purchased from the company. We rapidly discovered that the Stratagene plasmid could be replicated *in vitro* by the DNA synthesome, and that the level of DNA replication could be optimized to that observed in the *in vitro* DNA replication assay employing another routinely used DNA template, pSVO+. The spontaneous mutation frequency of the unreplicated plasmid transfected into the XL1-Blue MRF' strain of E.coli was found to be less than 1x $10^{-10}$.

While this aspect of the project was being developed, our laboratory group isolated the multiprotein DNA replication complex from two genetically matched human breast cell lines, Hs578BSt (non-malignant) and Hs578T (malignant), and two other human breast cell lines, the MCF 10A (non-malignant) and MCF7 (malignant), using the method previously described by our lab for other mammalian cell lines (27,28,35,36). The DNA synthesome from each of these human breast cell lines is fully competent to complete full length, semiconservative, large T-antigen dependent *in vitro* DNA replication. This fact, as well as results described previously by our lab (27,28,35,36) suggest that all of the cellular protein activities necessary for *in vitro* SV40 DNA synthesis are present within the DNA synthesome isolated from the Hs578Bst, Hs578T, MCF 10A, and MCF7 human breast cell lines. Additionally, the requirements for SV40 DNA replication *in vitro* by the isolated human breast cell DNA synthesome are comparable to the requirements that have been observed with crude cell extracts from permissive cells (30); namely, the initiation of SV40 DNA synthesis is dependent on the presence of both large T-antigen and a functional SV40 replication origin sequence.

An initial assessment of the level and fidelity of DNA replication carried out by the DNA synthesome of each of the human breast cell lines suggests a direct relationship between the relative rate with which the DNA template is replicated by the DNA synthesome and the relative frequency of mutational sequence errors that are created in

the replicated DNA. Thus far, we have observed the level of DNA replication to be between 5-9 times higher in the replication reactions mediated by the breast cancer cell DNA synthesome than in those reactions mediated by the non-malignant breast cell DNA synthesome. The apparent increased rate of replication carried out by the breast cancer cell DNA synthesome correlates with increased (3-5.5 times) frequency of mutant (white) colonies observed in the bacteria transfected with the plasmid replicated by the breast cancer cell DNA synthesome than in the bacteria transfected with plasmid replicated by non-malignant breast cell DNA synthesome. Although, to date, we cannot yet describe the actual differences, in the breast cancer cell DNA synthesome and the normal breast cell DNA synthesome, responsible for the altered replication rate and replication fidelity of the breast cancer cell DNA synthesome, it is likely that alteration in specific components of the DNA synthesome are responsible for these differences.

I have been expanding my initial analysis of the fidelity of the breast cancer cell DNA synthesome and the non-malignant breast cell DNA synthesome in order to develop an expanded pool of bacterial mutants expressing defects in the β-galactosidase gene. This pool of mutants will permit us to determine whether the defects in the β-galactosidase gene occur randomly or are localized to specific segments of the gene.

To date, I have begun to establish some of the types of mutations that have occured in the defective β-galactosidase genes collected from the mutant bacterial colonies selected in our assay. As shown in figure 10 (photo) and in figure 11, the types of mutations detected by our forward mutagenesis assay include invertions of T, and G-T and C-T mispairs.

Further sequencing of the β-galactosidase gene from mutant and normal (blue) colonies that were transfected with DNA from the other breast cancer cell line DNA synthesome-mediated replication reactions is in progress in our laboratory. We are also in the process of sequencing the β-galactosidase genes extracted from mutant and normal colonies transfected with the non-malignant DNA synthesome-mediated replication products.

This type of information, such as the appearance of mutation types characteristic of errors in proof-reading, will increase our understanding of the types of changes which may distinguish the cancerous breast cell DNA synthesome from the normal breast cell DNA synthesome.

To date, our results support the hypothesis that the cellular transformation process is, at least partially, a consequence of an increased rate in the accumulation of certain types of mutations and that these mutations arise, in part, due to a decrease in the fidelity of the DNA replication machinery (i.e. DNA synthesome) of the cancer cell.

Our additional experiments to measure the nucleotide excision DNA repair in the MCF 7 and MCF 10 A cell lines resulted in the unexpected discovery that the nucleotide excision repair activity of this breast cancer cell line is much greater than normal (25 fold higher). This is very surprising since there is considerable literature reporting the general decrease in DNA repair and DNA repair efficiency in cancer cells. It is also widely

accepted that there is a general increase in the level of DNA mutations in cancer cells. Perhaps this cancer cell line contains changes in other types of DNA repair mechanisms which decease, rather than enhance the DNA repair activity. The degree of infidelity with which the DNA is replicated by this cell line may also play a role in the increased incidence of mutations. Perhaps the increased DNA replication infidelity in MCF 7 (compared to MCF10A) cells may require a type of DNA repair other than nucleotide excision repair (such as mismatch repair). It is also possible that the other DNA repair systems in the cell are not equipt to compensate for the greatly enhanced number of errors produced by the cancerous DNA synthesome during replication.

Additional nucleotide excision repair assays are planned to investigate the DNA repair status of the Hs578Bst and Hs578T cell lines. We are also developing a collaboration in order to investigate the mismatch DNA repair activity of all four breast cell lines. These future investigations will help us to reach a more complete understanding of the role DNA replication fidelity plays in the overall fidelity of DNA in the human breast cancer cell.

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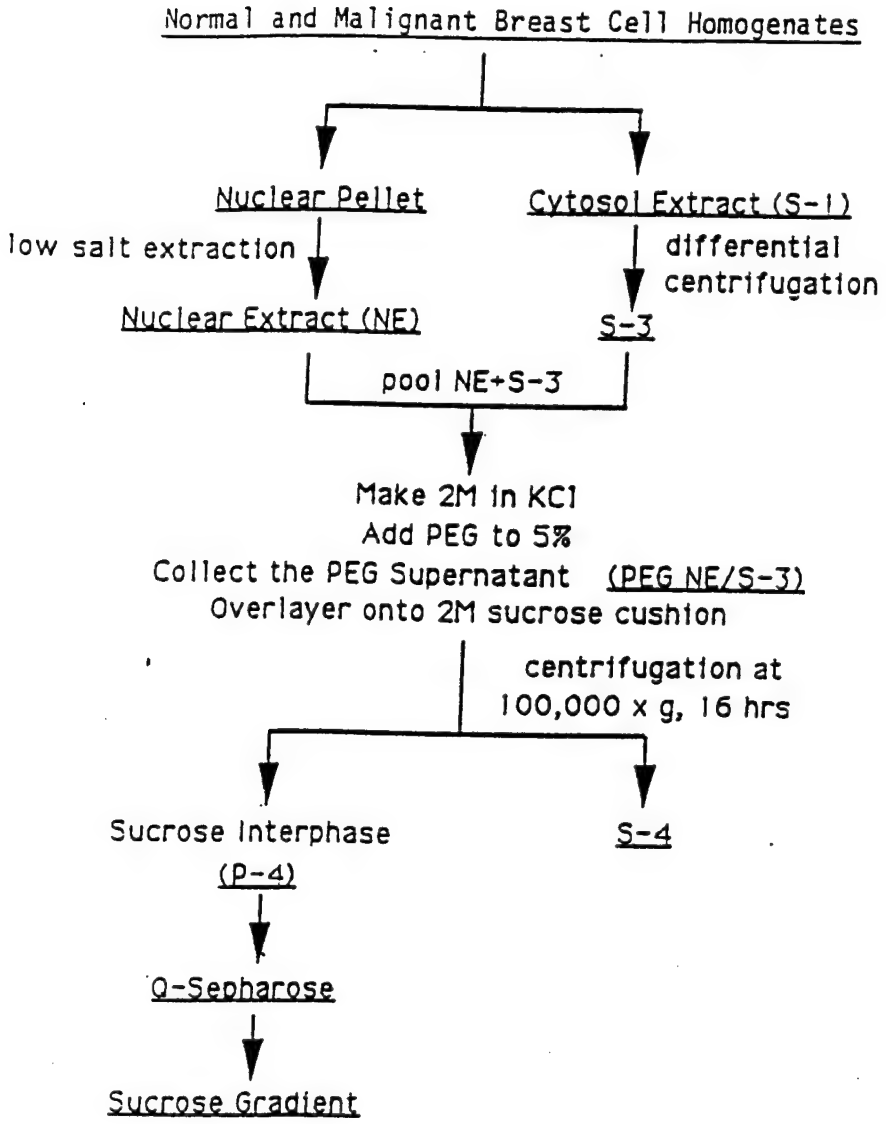

Normal and Malignant Breast Cell Homogenates
Nuclear Pellet
Cytosol Extract (S-1)
low salt extraction
differential centrifugation
Nuclear Extract (NE)
S-3
pool NE+S-3
Make 2M in KCl
Add PEG to 5%
Collect the PEG Supernatant (PEG NE/S-3)
Overlayer onto 2M sucrose cushion
centrifugation at 100,000 x g, 16 hrs
Sucrose Interphase (P-4)
S-4
Q-Sepharose
Sucrose Gradient

# Current Model for the MRC

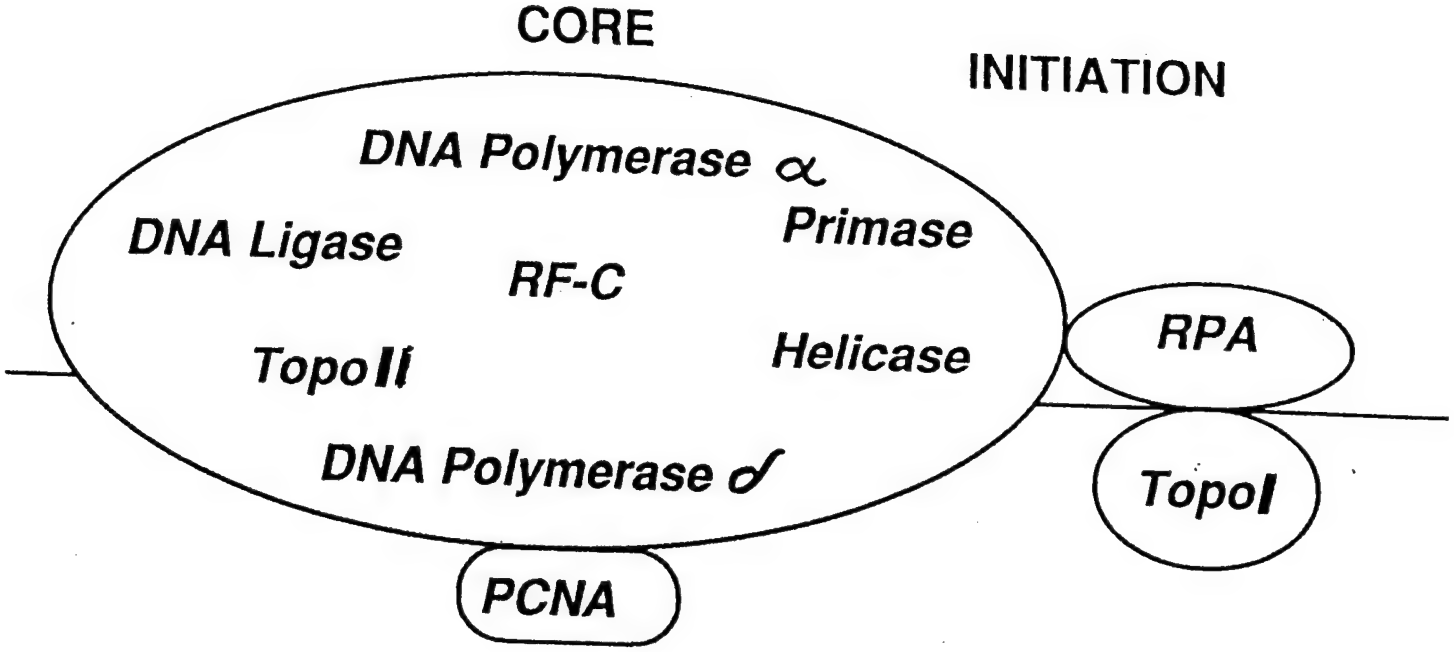
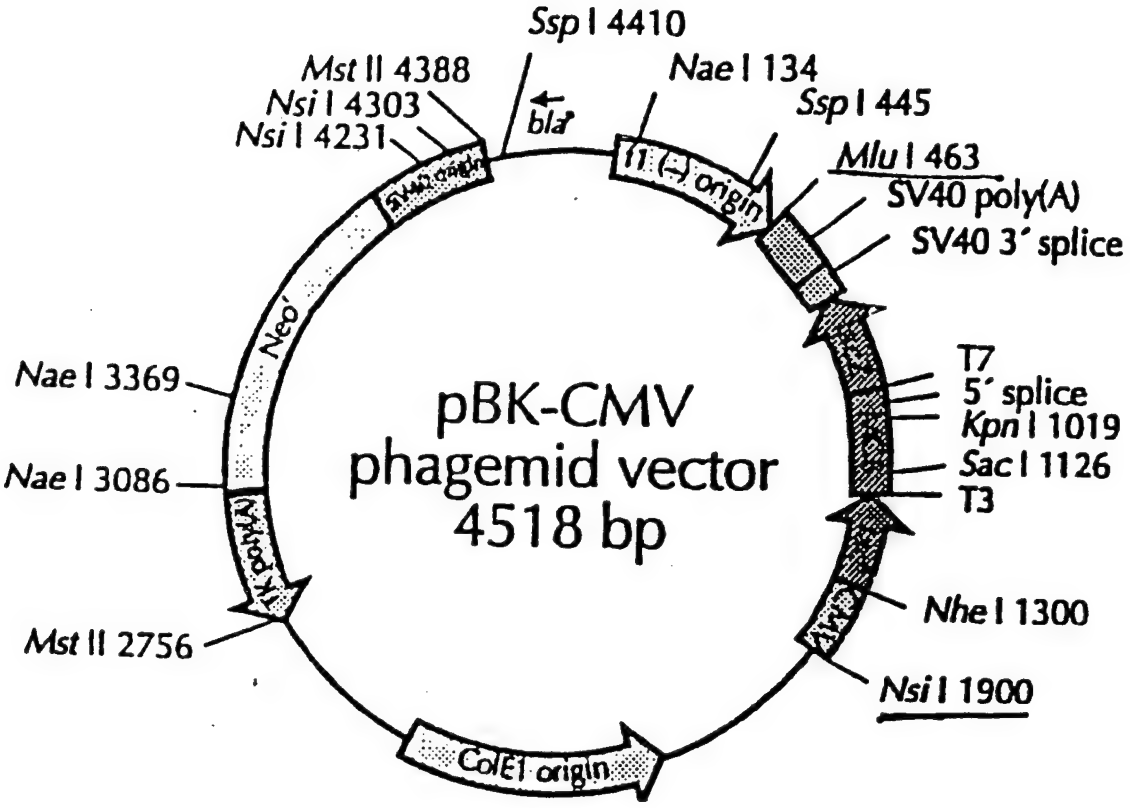
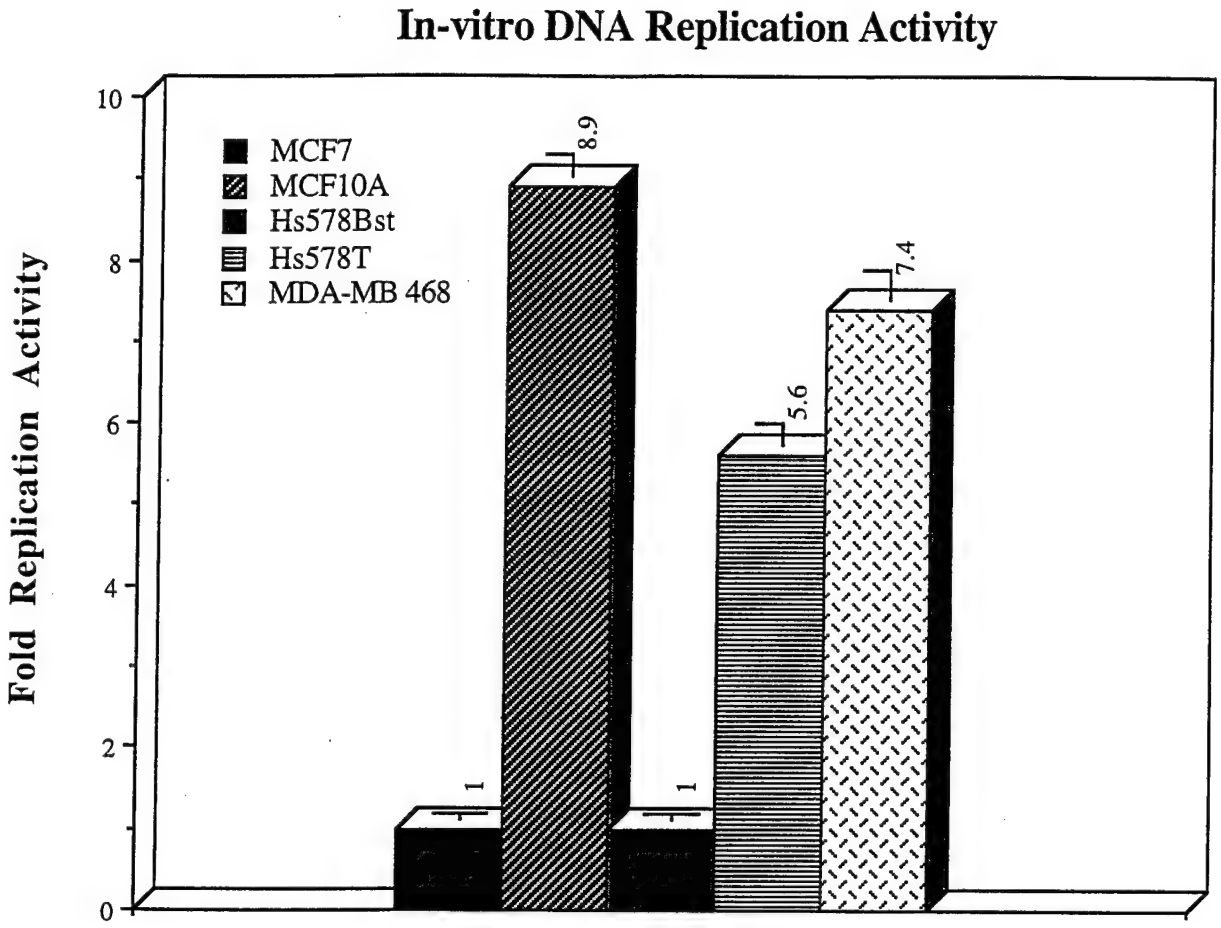





Figure 2

**Figure 3**


Ssp I 4410
Mst II 4388
Nsi I 4303
Nsi I 4231
bla
Nae I 134
Ssp I 445
Mlu I 463
SV40 poly(A)
SV40 3′ splice
T7
5′ splice
Kpn I 1019
Sac I 1126
T3
Nhe I 1300
Nsi I 1900
ColE1 origin
Mst II 2756
Nae I 3086
Nae I 3369
Neo'
pBK-CMV
phagemid vector
4518 bp


In-vitro DNA Replication Activity
Fold Replication Activity
10
8
6
4
2
0
MCF7
MCF10A
Hs578Bst
Hs578T
MDA-MB 468
1
8.9
1
5.6
7.4

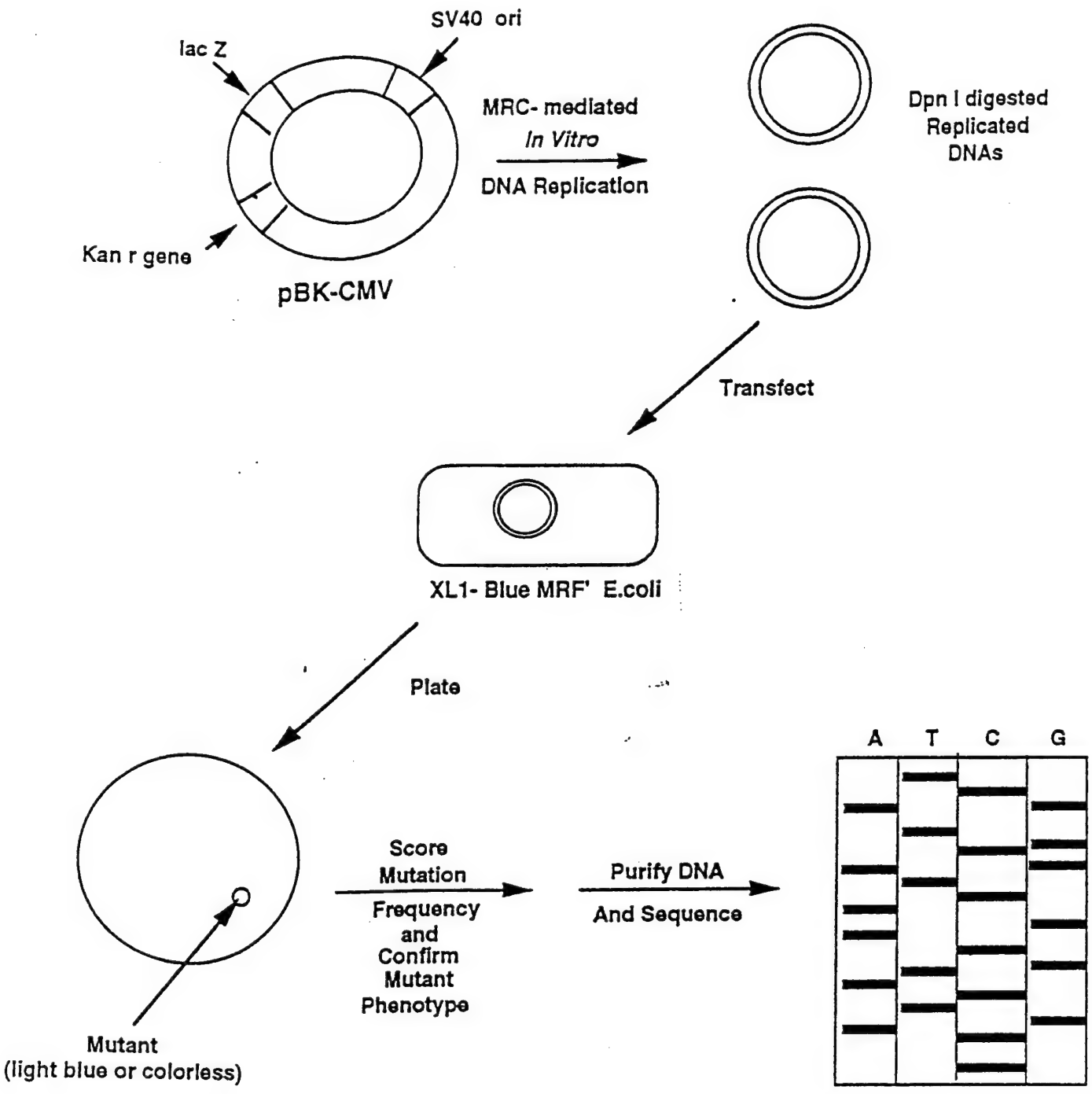
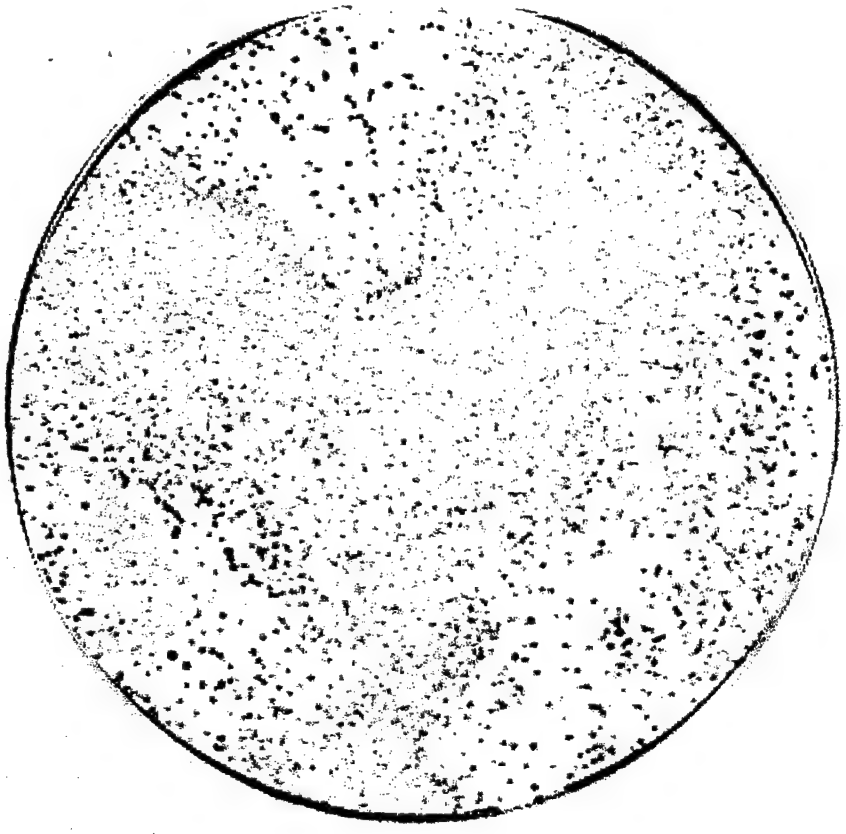
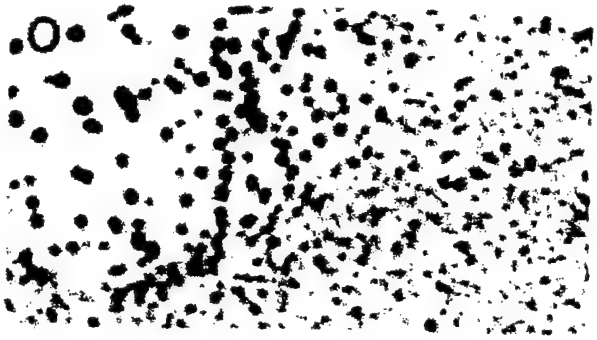

FORWARD MUTAGENESIS ASSAY: PART I
SV40 ori
lac Z
Kan r gene
pBK-CMV
MRC- mediated
In Vitro
DNA Replication
Dpn I digested
Replicated
DNAs
Transfect
XL1- Blue MRF' E.coli
Plate
Mutant
(light blue or colorless)
Score
Mutation
Frequency
and
Confirm
Mutant
Phenotype
Purify DNA
And Sequence
A
T
C
G

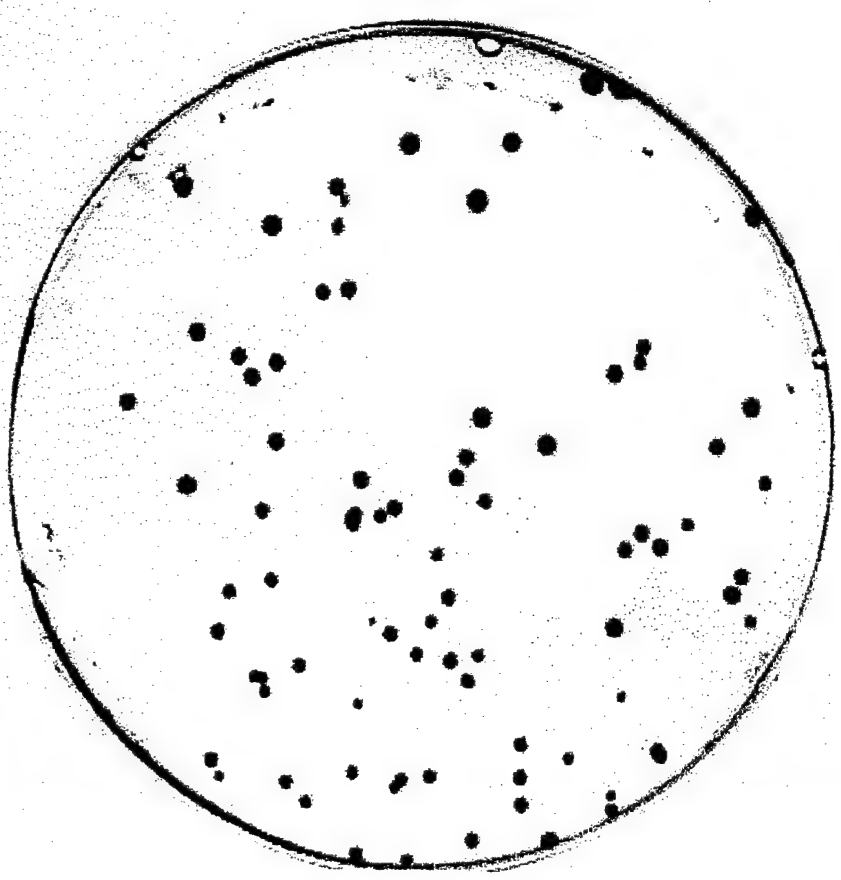
Figure 6



# Hs578Bst

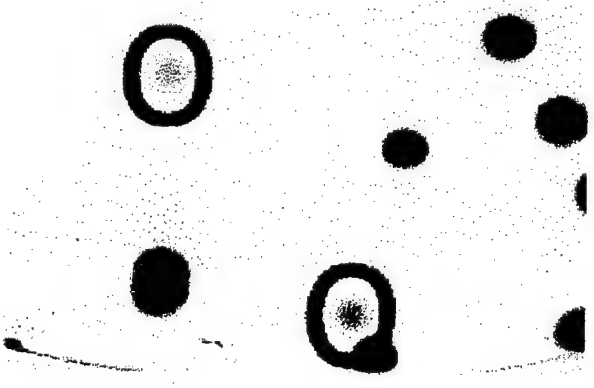
Mutation Rate: x=0.19% (N=3)
Enlarged to show detail

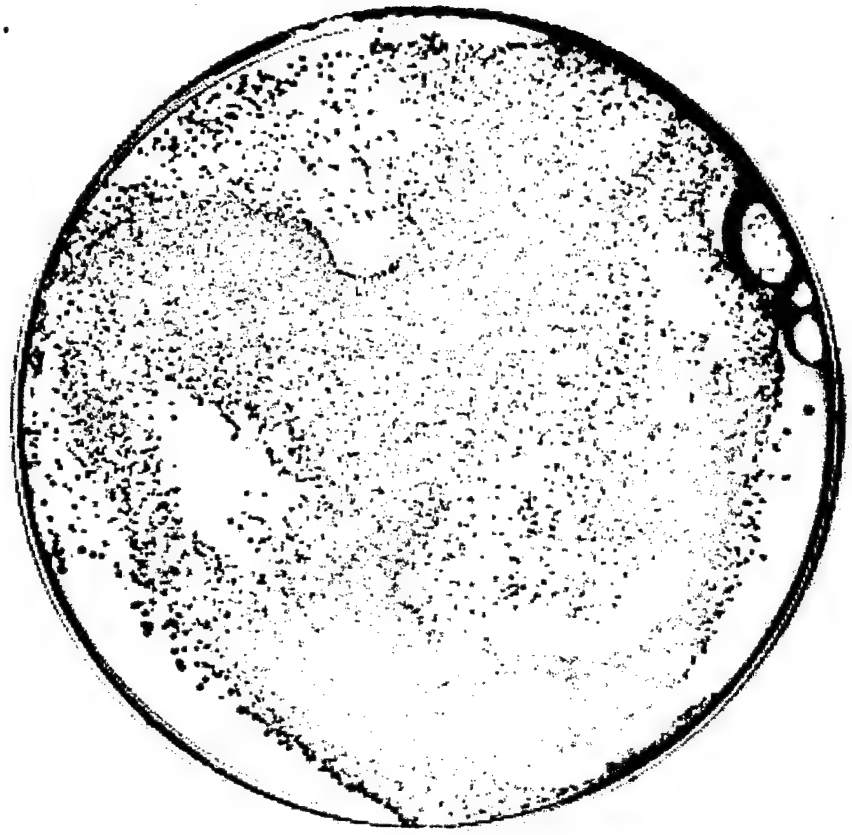




# Hs578T

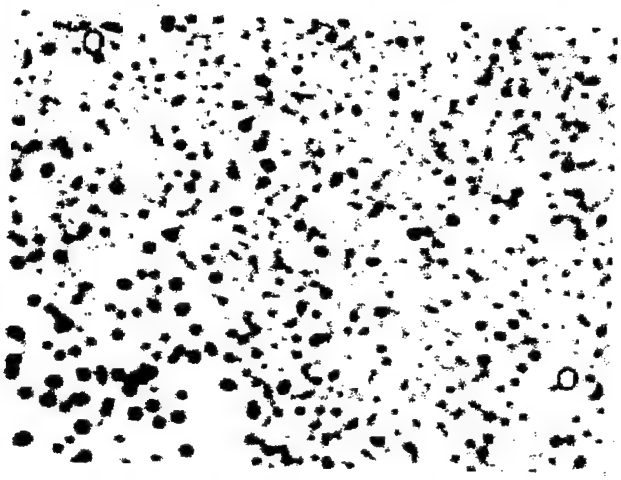
Mutation Rate: x=1.6% (N=3)
Enlarged to show detail

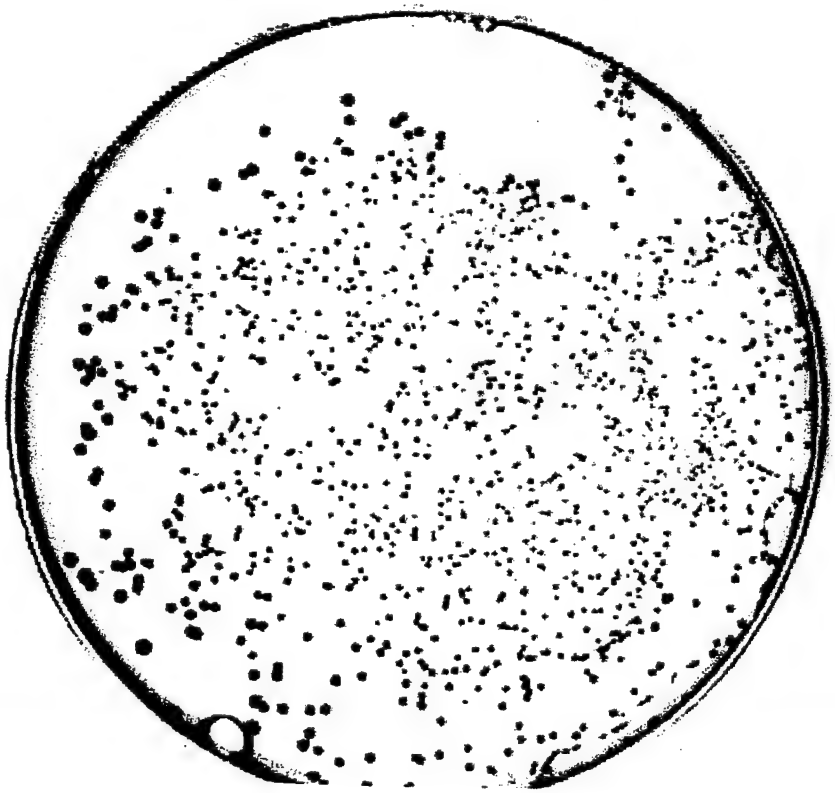




# MCF 10A

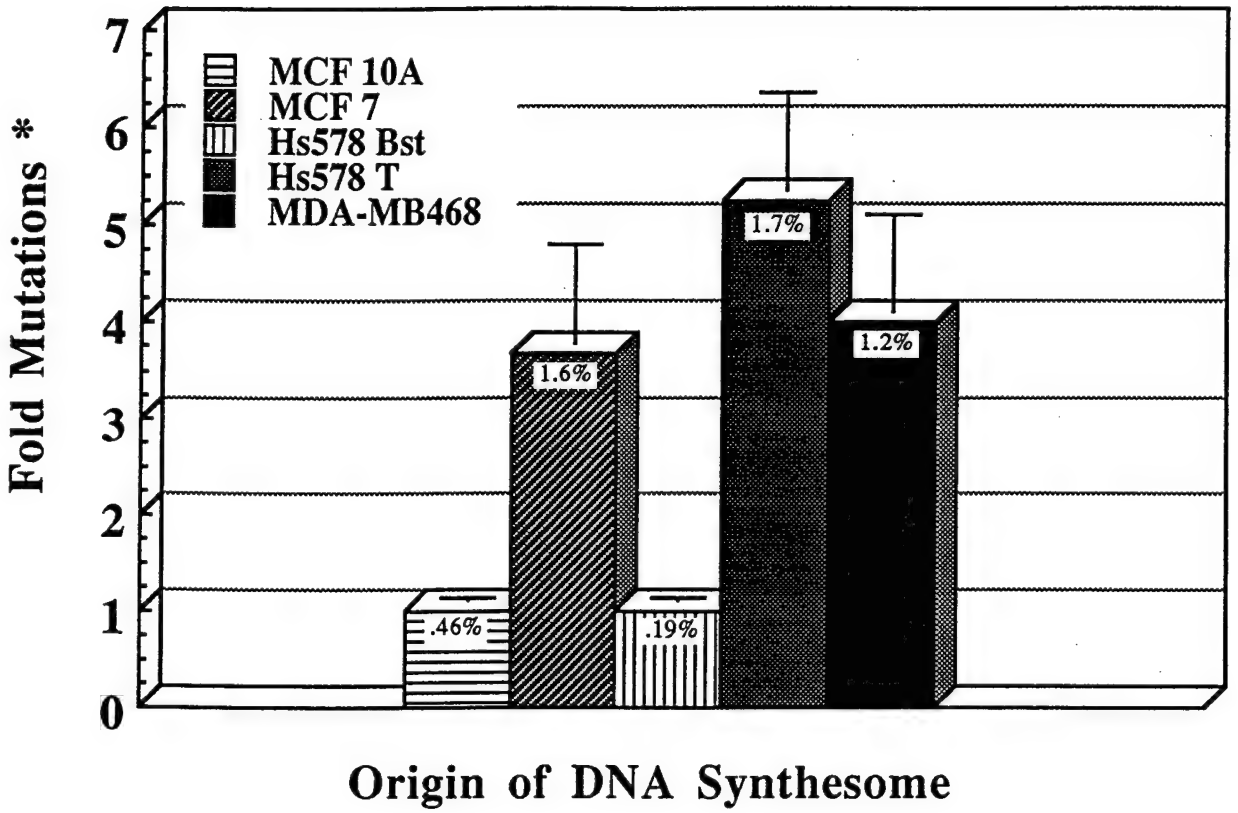
Mutation Rate: x= 0.46% (N=3
Enlarged to show detail





# MCF 7

Mutation Rate : x = 1.7 % (N=3)
Enlarged to show detail


Fold Mutations *
7
6
5
4
3
2
1
0
MCF 10A
MCF 7
Hs578 Bst
Hs578 T
MDA-MB468
.46%
1.6%
.19%
1.7%
1.2%
Origin of DNA Synthesome

## Sequencing primers (27bp each) for the α-lacZ gene

**Primer 1**

***5' CACTTAAAATTGGATCTCCATTCGCC (TOP STRAND)*** ----------->
3' GTGAATTTT AAC CTAGAGG TAAGCGG TAAGTCCGACGCGTTGACAA

*TOP STRAND---->* ----------->
3'GGGAAGGGCGTCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGG

---------->
3'GGATGTGCTGCAAGGCGATTAAGTTGG GTAACGCCAG GGTTTTCCCA

---------->
3'GTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTAT

---------->
3'AGGGCGAATTGGGTACACTTACCTGGTACCCCACCCGGGTGGAAAATCGA

---------->
3'TGGGCCCGCGGCCGCTCTAGAAGTACTCTCGAGAAGCTTTTTGAATTCTT

---------->
3'TGGATCCACTAGTGTCGACCTGCAGGCGCGCGAGCTCCAGCTTTTGTTCC

***5'— GAAATCACTCCCAATTAAA GCTC GAACC GCAT TAGTT CC A 3'***
3' CTTTAGTGAGG <---------**CGAGCTTGGCGTAATCAAGGT**

***5' ATA GATA CT GG***
**TAT CGATGACC 5' BOTTOM STRAND**

**Primer 2**

**Figure 10**


GATC
wt
GATC
mt
Note:
All sequenced DNA was from a replication reaction mediated by the MCF 7 DNA synthesome.
Key:
mt1= mutant (0+) phenotype
mt2= mutant (0+) phenotype
wt= wild type (4+) phenotpe
ct= control DNA (pUC 19)
[ ]= insertion in the G lane
: : = deletion in the G lane
| | = wt band in the T lane
< > = deletion in the T lane

# Mutations in the α lac Z gene of pBK-CMV Replicated by the MCF7 DNA Synthesome

*5' CACTTAAAATTGGATCTCCATTCGCC (TOP STRAND)* ——————>
3' GTGAATTTTAACCTAGAGGTAAGCGGTAAGTCCGACGCGTTGACAA

T to C mispair A :T transversion?
*TOP STRAND----->* ——— ——————————C————————— T————————>
3'GGGAAGGGCGTCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGG

insertion of additional C
———————————————— ACCCCATT ——————————————>
GGGATGTGCTGCAAGGCGATTAAGTTGGGTAA CGCCAG GGTTTTCCCA

T to G mispair
————————————————G ——————————————————————>
3'GTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTAT

T to G mispair T: A trnasversion?
————————G ——————————————————————A————————————>
3'AGGGCGAATTGGGTACACTTACCTGGTACCCCACCCGGGTGGAAAATCGA

T to G mispair
————————————————————————————————————G ——————————>
3'TGGGCCCGCGGCCGCTCTAGAAGTACTCTCGAGAAGCTTTTTGAATTCTT

deletion of TA
—— " TA"————————————————————————————————————————>
3'TGGATCCACTAGTGTCGACCTGCAGGCGCGCGAGCTCCAGCTTTTGTTCC

*5'— GAAATCACTCCCAATTAAA GCTC GAACC GCAT TAGTT CC A*
3' CTTTAGTGAGG <--------- **CGAGCTTGGCGTAATCAAGGT**

*5' ATA GATA CT GG*
3' **TAT CGATGACC** **5' BOTTOM STRAND**

# DNA Nucleotide Excision Repair Assay